Title: An Efficient and Cost-effective Method of Generating Postnatal (P2 - 5) Mouse Primary Hippocampal Neuronal Cultures

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Abstract

Background

Primary culture of postnatal central neurons is a widely used methodology for applications such as the investigation of neuronal development, protein trafficking/distribution and cellular signalling. However, successful production and maintenance of such cultures, particularly from postnatal animals, can be challenging. In attempting to surmount these difficulties, several disparate culturing methodologies have been developed. Such methodologies are centred on the identification and optimisation of critical steps and, as such, the protocols and reagents utilised can differ quite markedly from protocol to protocol, often with the suggestion that the use of a (usually expensive) proprietary reagent(s), lengthy substrate preparation and/or cell isolation techniques is/are necessary for successful culture preparation.

New Method

Herein, we present a simple and inexpensive protocol for the preparation of primary hippocampal neurons from postnatal (2 - 5 day old) mice, which remain viable for experimental use for over one month.

Results

Neurons cultured using this method follow well established developmental norms and display typical responses to standard physiological stimuli such as depolarisation and certain pharmacological agents.

Comparison with Existing Methods/Conclusion

By using a novel trituration technique, simplified methodology and non-proprietary reagents, we have developed a reliable protocol that enables the cost effective and efficient production of high quality postnatal mouse hippocampal cultures. This

method, if required, can also be utilised to prepare neurons both from other regions of the brain as well as from other species such as rat.

Keywords

Primary neuronal culture; Hippocampal neurons; Postnatal; Trituration; Mouse.

Abbreviations

Postnatal (P), (S)-3, 5-dihydroxyphenylglyccine ((S)-DHPG), N-methyl-D-aspartic acid (NMDA), days *in vitro* (DIV), group I metabotropic glutamate receptor (mGluR).

1. Introduction

1.1 Uses of primary neuronal culture

Primary neuronal culture enables the conversion of complex, three dimensional brain tissue, which is difficult to study *in vivo,* into a two-dimensional monolayer of cells, which allows easy access to, and visualisation of, individual neurons and synapses (Nunez, 2008). As such, the technique is routinely utilised for a wide variety of applications in neuroscience including, *inter alia,* pharmacological, electrophysiological, immunohistochemical, neurotoxicological, developmental and cell signalling studies (Beaudoin et al., 2012; Brewer and Torricelli, 2007; Nunez, 2008). However, in addition to ease of access to, and visualisation of, neurons and synapses, this simplified monolayer system is also of use for several other purposes. For example, neuronal cell culture eliminates, or at least reduces, potential hormonal, vascular and/or inflammatory confounding influences that one might encounter when using intact, or whole brain tissue, systems (Brewer and Torricelli, 2007), which may be important when investigating phenomena such as the subcellular localisation and trafficking of neuronal proteins such as neurotransmitter receptors (Chen et al., 2008). Conversely, one can easily and very specifically manipulate the cellular environment of cultured neurons/glia to determine the effect of a particular pharmacological or hormonal intervention in the absence of any other confounding factors. Thus, careful and selective manipulation of the cellular environment in this way provides more specific and reproducible information than could be achieved by using whole tissue or *in vivo* animal studies in the same way (Zhang et al., 2006).

Neuronal culturing also enables the preparation of multiple, separate neuronal ‘populations’ which can be considered as identical replicates (Brewer and Torricelli,

2007). As it is usually possible to seed a relatively large number of identical neuronal populations from a single piece of brain tissue, this technique represents a highly efficient method of increasing sample size, whilst reducing variability. Finally, but no less importantly, given the *relative* long-term viability of cultured cells compared with acutely dissected tissue such as acute brain slices, cell culture also allows for longer term studies investigating toxicology and development (Chen et al., 2008; Zhang et al., 2006).

1.2 Embryonic versus postnatal brain tissue

The possible advantages of using cultured neurons/glia over intact brain tissue notwithstanding, there can be significant practical limitations to producing viable neuronal cultures as the technique itself is technically demanding and can be hampered by a lack of reproducibility.

Variation in the quality of neuronal cultures can be ameliorated to some extent by utilising embryonic, rather than postnatal, tissue. This is most likely due to the proposed increased plasticity of embryonic, relative to postnatal, neurons. As such, they have fewer complex neurites, lower inter-neuronal connectivity and a decreased reliance on trophic support (Brewer and Torricelli, 2007; Kivell et al., 2000; Zhang et al., 2006). Taken together, these factors are likely to bestow upon embryonic cultures a higher resistance to, and higher survival rate following, chemical and mechanical tissue dissociation. This technical advantage has led to the widespread use of embryonic tissue, particularly that of mice, for the preparation of neuronal cultures. Although, the use of embryonic neuronal culture may be relevant for studies concerned with, for example, neuronal/glial development, disease etiology, or where, due to genetic mutation(s), animals die at, or soon after, birth, it would often be much

more appropriate to use more physiologically-relevant, postnatal tissue. For example, when investigating the function and role of developed neurons, and for studies linked to age-developed pathology such as Alzheimer's disease. Unfortunately, however, in contrast to embryonic tissue, postnatal brain tissue is relatively sensitive to the culturing process. This is thought to be due to the fact that as neurons develop, they become more susceptible to glutamate-mediated excitotoxicity (Brewer, 1998) and exhibit enhanced caspase activation and apoptosis (Brewer et al., 2005). Physical degradation of neurons also removes intra-neuronal trophic support, thereby decreasing cell viability still further (Brewer and Torricelli, 2007).

This susceptibility to culturing seems to be particularly true when one considers the culturing of hippocampal and other central neurons derived from postnatal *mice*, where there are limited documented successful culturing methodologies for either very early postnatal (postnatal day (P) 0 - 1) (Ahlemeyer and Baumgart-Vogt, 2005; Beaudoin et al., 2012) or adult mice (Brewer and Torricelli, 2007; Eide and McMurray, 2005), relative to protocols published for producing postnatal rat neuronal cultures (e.g. (Brewer, 1997; Drysdale et al., 2006; Hogins et al., 2011; Irving et al., 2000; Kivell et al., 2000; Nunez, 2008; Rae et al., 2000; Rao and Sikdar, 2004; Stoppelkamp et al., 2010; Ternaux and Portalier, 1993; Zhang et al., 2006). Given that the vast majority of transgenic animal models that have been developed to date are mice, this is a very significant issue. Thus, a reliable and cost-effective postnatal mouse primary hippocampal culturing protocol could have widespread applications, particularly in the fields of learning and memory, and neurodegeneration.

1.3 Previous studies

To this end, to date, opting to use postnatal tissue to prepare primary neuronal cultures often “means accepting the fact that there will be some bad culture days” Banker et al. (2007). Such “bad cultures” and lack of reproducibility are likely to arise from subtle variations in reagents used (Beaudoin et al., 2012) and/or techniques employed between labs. This has resulted in the utilisation and publication of several distinct culturing protocols, with each containing highly specific, often very different, and sometimes even conflicting, advice for each facet of the culturing protocol. However, differences in protocol notwithstanding, a review of recent literature reveals the widely held belief that preparation of postnatal mouse cultures specifically necessitates the use of expensive, defined media and supplements, namely, Neurobasal®-A (NB-A) and the HEPES-based Hibernate®A, which are utilised in combination with a proprietary supplement solution, B-27® serum-free supplement (B-27) (Ahlemeyer and Baumgart-Vogt, 2005; Beaudoin et al., 2012; Brewer, 1997; Brewer and Torricelli, 2007; Drysdale et al., 2006; Eide and McMurray, 2005; Hui et al., 2015; Kivell et al., 2000; Nunez, 2008; Rao and Sikdar, 2004; Stoppelkamp et al., 2010; Xie et al., 2000; Zhang et al., 2006). Indeed, based upon a review of recent literature on the topic, one would be forgiven for thinking that it is nigh on impossible to prepare postnatal cultures without using these proprietary reagents and supplements (as well as lengthy protocols such as gradient separation to further optimise the procedure (Brewer & Torricelli, 2007). However, not only are these proprietary media and supplements much more expensive than standard, non-proprietary, media preparations such as Dulbecco’s modified Eagle’s medium (DMEM), there is also a concern in some quarters about variable quality and uniformity between batches of B-27 (Chen et al., 2008) and certain, possibly neurotoxic, ingredients contained within NB-A (Hogins et al., 2011).

159 Therefore, it was the aim of the present study to determine the possibility of
160 producing consistent, successful postnatal primary mouse hippocampal neuronal
161 cultures utilising non-proprietary solutions and supplements whilst, at the same time,
162 also attempting to simplify the overall culturing protocol relative those published in
163 recent times (e.g. Brewer & Torricelli, 2007, Beaudoin et al., 2012) (Suppl. Table 1
164 provides a comparison of the main components of previously published postnatal
165 mouse hippocampal culture methods with our method).
166 Herein, we describe a simplified, technically straightforward and reliable method for
167 the production of postnatal (P2 - P5) mouse hippocampal cultures of consistently
168 high quality which remain viable and responsive to normal physiological stimuli for
169 over one month post-culture. We show that the critical element in the production of
170 successful neuronal cultures is the deployment of a novel, mild, tissue trituration
171 protocol which produces sufficient neuronal dissociation without overly deleterious
172 effects on neurons or the production of excessive amounts of cellular debris.
173 Significantly, good quality cultures can be produced using this method without the
174 need for the aforementioned expensive, proprietary media and supplement.

2. Materials and Methods

2.1 Chemicals & Reagents

- Mice (2 – 5 days old)
- Serum replacement 2 (SR2) (50×) (Sigma, cat. no. S9388).
- Glutamax™ supplement (ThermoFisher Scientific, cat. no. 35050038).
- L-glutamic acid (Sigma, cat. no. G1251).
- Penicillin-streptomycin (P-S) (Sigma, cat. No. P4333).
- Foetal bovine serum (FBS) (Sigma, cat. no. F7524). Storage limitations: store at -80ºC and limit freeze thaw cycles to one.
- Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma, cat. no. D5546).
- Papain (Worthington, cat. no. 3119) **(see *Reagent Setup*).**
- Poly-D-lysine hydrobromide (Sigma, cat. no. P6407) **(see *Reagent Setup*).**
- Dissection, trituration, plating and culture solutions **(see *Reagent Setup*).**
- Trypan blue solution (Sigma, cat. no. T8154) (optional).
- 70% ethanol solution for sterilisation of surfaces and hands.
- Deionised water (dH_2O).
- Immunocytochemistry reagents: Triton™ X-100 (Sigma, cat. no. X100), paraformaldehyde (PFA) (Sigma, cat. no. P6148), sucrose (Sigma, cat. no. S7903), glycine (e.g., Sigma, cat. no. G7126), bovine serum albumin (BSA) (Sigma, cat. no. 05470), mounting medium (Sigma, cat. no. F6182), microscope slides (25 x 75 mm) (Sigma, cat. no. S8400). Primary antibodies: pan neuronal marker (mouse monoclonal, Millipore, cat. no. MAB2300, Antibody Registry: AB_1587299), anti-GFAP (rabbit monoclonal, Cell Signaling, cat. no. 12389, Antibody Registry:

AB_2631098), anti-Synapsin I (rabbit polyclonal, Millipore, cat. no. AB1543, Antibody Registry: AB_2200400), anti-NeuN (mouse monoclonal, Millipore, cat. no. MAB377, Antibody Registry: AB_2298772). Secondary antibodies: FITC goat anti-mouse (Jackson, cat. no. 115-095-003, Antibody Registry: AB_2338589), Cy5 goat anti-rabbit (Abcam, cat. no. ab97077, Antibody Registry: AB_10679461), TRITC donkey anti-rabbit (Jackson, cat. no. 711-025-152, Antibody Registry: AB_2340588).

- Drugs used for calcium imaging: (S)-3, 5-dihydroxyphenylglyccine ((S)-DHPG) (Tocris, cat. no. 0805), caffeine (Sigma, cat. no. C0750), carbachol (Tocris, cat. no. 2810), N-methyl-D-aspartic acid (NMDA) (Sigma, cat. no. M3262)

2.2 EQUIPMENT

- 35 mm cell culture dish (untreated) (Sigma, cat. no. CLS430588). Previous studies have suggested cell culture treated dishes give rise to altered neuronal morphology (Chen et al., 2011).
- 145 mm cell culture dish (Sigma, cat. no. Z652539)
- 13 mm glass coverslips, other sizes can be used as desired (TAAB, cat. no. M160/1).
- 15 and 50 ml sterile polypropylene (PP) centrifuge tubes (Fisher Scientific, cat. no. 11849650 and 11512303 respectively). Previous studies have recommended the routine use of polystyrene or polyethylene terephthalate (PET) tubes given reports of toxicity from PP tubes (Brewer and Torricelli, 2007), but we found no adverse effects from use of PP tubes.
- 15 ml and 30 ml syringes (Fisher Scientific, cat. no. 12931031and 10313015 respectively)
- Haemocytometer (optional).

- Sterile syringe filter, 0.2 µm pore size (Sigma, cat. no. CLS431229).
- 150 mm Pasteur pipette (VWR, cat. no. 612-1701) **(see *Equipment Setup*).**
- 3.5 ml Plastic transfer pipette (Sarstedt, cat. no. 86.1171).
- Incubator, controlled 5% CO_2, humidified. Other studies have recommended the use of an O_2 regulated incubator (Brewer and Torricelli, 2007). However, in our experience these more costly incubators are not necessary for producing successful cultures.
- Inverted microscope, phase contrast, with long working distance objective.
- Sterile laminar flow hood, HEPA-filtered air.
- Sterile dissection hood, HEPA-filtered air.
- Swinging bucket centrifuge compatible with the use of 15 ml tubes, ambient room temperature, capable of speeds of 258 g.
- Water bath.
- Temperature-regulated orbital shaker, capable of incubating at 37 ^{O}C.
- Fine and course dissection tools.
- Osmometer.

2.3 REAGENT SETUP

- **HEPES-buffered saline solution (HBSS)** of the following composition (in mM): NaCl 130, HEPES 10, KCl 5.4, $MgCl_2$ 2, D-glucose 2 and $CaCl_2$ 0.5, pH 7.4, 310 mOsm (chosen to match the range of osmolarity (304-336 mOsm) of DMEM).
- **Dissection solution.** Prepare 8 ml of HBSS solution containing 0.5 mM Glutamax, 0.025 mM L-glutamic acid, 1% P-S.
- **Trituration solution.** Prepare 15 ml of HBSS solution containing 10% FBS, 5 mM Glutamax, 0.025 mM L-glutamic acid, 1% P-S. Place in water bath at 37ºC.

- **Plating solution.** Prepare 2 ml of DMEM solution containing 10% FBS, 5 mM Glutamax, 0.025 mM L-glutamic acid, 1% P-S. Place in incubator and allow CO_2 to buffer solution.
- **Culture solution.** Prepare 35 ml of DMEM solution containing 2% SR2, 5 mM Glutamax, 0.025 mM L-glutamic acid, 1% P-S. Place in incubator and allow CO_2 to buffer solution.
- **Poly-D-lysine solution**. Dissolve at 0.1 g/ml in dH_2O. Store as frozen 2 ml aliquots in 15 ml tubes.
- **Papain solution.** Immediately prior to beginning dissection, dissolve 2.5 mg/ml papain in Dissection solution with a total volume of 2 ml (approximately 25 U ml^{-}1). To ensure full dissolution of papain, the solution should be placed in a 37°C water bath for approximately four minutes. Filter sterilise the solution into a 15 ml tube and store on ice. Note: Papain has been found to be the most suitable protease for cell culture (Brewer, 1997). Although in our hands papain from Worthington Scientific was superior to that purchased from Sigma, the specific supplier does not appear to be crucial for culture success. The merits of dissolving papain in Ca^{2+} and Mg^{2+} free solution, to aid tissue digestion (Copray and Liem, 1993) are debated, due to the damage that an absence of these cations may have directly on cells (Kivell et al., 2000). It is recommended that papain be stored on ice for no longer than three hours (Brewer and Torricelli, 2007).
- **Phosphate Buffered Saline (PBS)** of the following composition (in mM): NaCl 137, KCl 2.7, NA_2HPO_4 10, KH_2PO_4 2, pH 7.4.
- **Mice (2–5 days old).** The volumes provided in this protocol are designed for hippocampi from three to four mice aged between 2–5 days old and would have to be adjusted accordingly if one wished to use older animals. Animals were euthanized

in accordance with the European Directive 2010/63/EU and experiments approved by the Animal Experimentation Ethics Committee of University College of Cork.

2.4 Equipment Setup

2.4.1 150 mm Pasteur pipette

Select pipettes with smooth tips and flame polish briefly (approximately two seconds) to blunt, but not significantly reduce aperture size. Note: The use of siliconised glass is recommended by some in the field for ease of trituration and transfer due to the viscous nature of the dissociated cell suspension (Brewer and Torricelli, 2007). However, in our hands standard glass pipettes were sufficient.

2.4.2 Preparation of coverslips

In a laminar flow hood, coverslips are transferred to a 35 mm cell culture dish containing 2 ml of 70% ethanol for sterilisation. Two coverslips are then removed and placed upright in each of sixteen 35 mm cell culture dishes and allowed to air dry. Once dried, coverslips are placed horizontally on the base of each 35 mm dish.

Note: Cell substrate cleaning and coating are key steps in the culture process which are utilised to ensure neuronal attachment and survival, as well as standard development and maturation (Kaech and Banker, 2006). In terms of substrate material, adult neurons do not grow well on plastic (Brewer and Torricelli, 2007) and as such, glass coverslips are routinely used. Moreover, there are recommended providers of cover slips with a proven reliability, without which cells will initially grow but detach soon after, such as: Type “D” glass from Schott Desag, (Kaech and Banker, 2006). In our hands, coverslips provided by TAAB have also been conducive

300 to long-term reliable culturing. Treating these substrates with nitric acid is
301 discouraged by some (Brewer, 1997), but recommended by others, including a
302 lengthy 'etching' procedure with nitric acid, followed by thorough cleaning with
303 distilled H_2O, and then heat and/or radiation sterilization (Beaudoin et al., 2012;
304 Kaech and Banker, 2006). In our hands however, quick sterilization in 70% ethanol
305 was sufficient to allow for poly-D-lysine attachment and subsequent cell adherence,
306 without any adverse effects on neuronal survival, despite the assertion that solvents
307 leave a ruinous toxic residue (Brewer and Torricelli, 2007).
308
309 *2.4.3 Poly-D-lysine coating of coverslips*
310 One drop of sterile filtered poly-D-lysine solution was added to the centre of each
311 coverslip. The 35 mm cell culture dishes were then placed into two larger "holding"
312 culture dishes (145 mm) and left at ambient room temperature. After one hour, the
313 poly-D-lysine was aspirated and the coverslips rinsed twice with a drop of dH_2O.
314 Timing: This step can be conducted during the tissue incubation with papain. Note:
315 poly-D-lysine is superior in terms of consistent cell attachment and neurite
316 outgrowth, when compared with poly-L-lysine (Kivell et al., 2000). In the interest of
317 efficiency, we opted not to make poly-D-lysine fresh for each dissection as
318 recommended by Kaech and Banker (2006) and instead used frozen stocks, whilst
319 limiting freeze-thaw cycles to no more than two (Brewer and Torricelli, 2007).
320 Coverslips may be prepared in advance of the day of tissue preparation and have
321 been shown to be stable for up to seven days at 4 $^{\circ}$C (Beaudoin et al., 2012).
322 Although serum pre-treatment of coverslips is reported as being essential for cell
323 attachment, presumably linked with an initial stimulus for neurite growth which
324 facilitates attachment (Kivell et al., 2000), we opted to provide this stimulus through a

serum-containing plating medium.

2.5 Methods

Unless otherwise stated, all procedures were carried out in a sterile laminar flow hood, using equipment and reagents which, if not already contained in sterile packaging, were sterilised with 70% ethanol. Strict aseptic technique must be adhered to in order to avoid contamination.

2.5.1 Solution preparation

Approximately 3 ml of dissection solution was sterile filtered into each of two 35 mm dishes which were placed on ice in a dissection hood.

2.5.2 Tissue isolation and treatment

Hippocampi were isolated as described previously (Beaudoin 2012) and placed in the 35 mm dish on ice. The tissue was cleaned before being transferring to the second 35 mm dish on ice. The tissue was then chopped into smaller pieces with fine scissors and then transferred to a 15ml tube using a Pasteur pipette. This tube was placed in a water bath at 37ºC with the papain solution, separately, and both were allowed to equilibrate at this temperature for five minutes. The tissue was then carefully transferred into the papain solution and placed on an orbital shaker at 100 rpm at 37ºC for 45 minutes. The tissue was then gently removed from the papain solution and placed into another 15 ml tube containing 2 ml HBSS. This step was repeated two more times (to remove any residual papain), each time being careful to avoid trituration of the tissue at this stage. The tissue was then transferred into another 15 ml tube containing approximately 6 ml of trituration solution.

2.5.3 Trituration (see accompanying video)

The tissue was allowed to settle to the bottom of the 15 ml tube containing the trituration solution. The tip of a plastic transfer pipette, with the bulb fully depressed, was firmly pushed against the bottom of the 15 ml tube. The solution containing the tissue was then aspirated into the pipette such that the tissue experienced a certain degree of friction as it moved between the edges of the bottom of the tube and the pipette itself. This friction helped to dissociate individual hippocampal neurons from the larger pieces. When all of the tissue had been aspirated into the pipette, the tip of the pipette was then placed under the meniscus of the trituration solution and forcefully expelled. Depending on the resulting tissue break up, this procedure was repeated up to an absolute maximum of four times, allowing the tissue to settle to the bottom of the tube between each aspiration. The supernatant was then placed into another 15 ml tube, leaving behind as much as possible of the untriturated tissue. This whole procedure should take no more than five minutes. The supernatant was then centrifuged at 258g for two minutes at room temperature. Note: The trituration procedure is the most critical step in determining the success of a culture. As this is the point in the protocol where cells experience the main mechanical stress, a balance is required between reaching optimal cell yield by sufficient tissue dissociation, whilst minimising cell lysis. Such lysis and debris production can both directly and indirectly, by altering the pH of the trituration solution and releasing cytotoxic agents such as glutamate, hinder cell attachment, sprouting and viability (Brewer, 1997; Eide and McMurray, 2005). Tip diameter is also crucial in determining overall cell yield, with too small a tip diameter causing cell damage, and too large a diameter resulting in insufficient breakdown of the tissue. We found that a plastic, wide tip diameter (drop size 35–55 µl) transfer pipette (see equipment) was optimal

for reaching this compromise. By using such a plastic pipette, the unreliable nature of creating an optimally smooth glass pipette tip, which can result in such a small a tip diameter that it damages cells, is circumvented (Beaudoin et al., 2012). Using this novel trituration approach, we do notice some air bubble production does occur. Although this is a phenomenon which has been actively discouraged by others (Brewer and Torricelli, 2007), we have found that it does not significantly affect the quality of our cultures in comparison to, say, over trituration which is much more damaging.

[insert trituration video here]

2.5.4 Cell seeding and culture

After centrifugation, the supernatant was poured out of the tube, being careful not to disturb the pellet of cells. Two drops of plating solution were then gently added and subsequently removed in order to dilute any residual supernatant. The volume of plating solution added (0.6 - 1 ml) is determined subjectively, depending on the size of the pellet which, in turn, is dependent on the effectiveness of trituration. This effectively results in a plating density from 1 – 1.5 x 10^5 cells/cm^2. The pellet was carefully dislodged from the bottom of the 15 ml tube using a Pasteur pipette, and the cells subsequently re-suspended by firmly finger vortexing 2 - 3 times, depending on the level of resuspension. At this point, if an inexperienced operator has difficulty objectively determining the amount of plating solution to be added, a sample can be taken for cell counting using a hemocytometer, and the density altered thereafter. An indication of successful technique up to this point can be determined by employing a trypan blue staining and cell counting protocol (as per manufacturer's instructions). A drop (approximately 25 μl) of the final plating solution was then added to each

coverslip using a Pasteur pipette. The coverslips were then placed in the incubator, where the cells were allowed adhere to the coverslips for one hour. Two ml of culture solution was then very gently added to each 35 mm dish (to prevent damaging the cells through sheer stress), before returning the dishes to the incubator for a further two hours, after which the coverslips were inverted using sterile forceps. In order to prevent cross contamination, forceps were flame sterilised between dishes**.**

2.5.5 Immunocytochemistry

Wash steps consisted of three changes of PBS, each lasting five minutes. Cells were fixed with paraformaldehyde (4 %) & sucrose (4 %) in PBS, warmed to 37^{o}C for ten minutes. All subsequent steps, unless otherwise stated, were carried out at room temperature. After washing, a permeabilisation solution (0.25 % Triton™X-100 in PBS) was added for ten minutes. Cells were washed once more with PBS and a blocking buffer (BSA 5 % & Glycine 0.3 M in PBS) was added for one hour. Primary antibody incubation was carried out overnight at 4^{o}C using the following antibodies: Pan Neuronal Marker (1: 100), GFAP (1: 500), Synapsin I (1: 200). All antibodies were diluted in an antibody buffer containing BSA 1 % in PBS. After washing, cells were incubated in the corresponding secondary antibody at 1: 200 dilution for one hour. Cells were again washed before being mounted on microscope slides.

2.5.6 Determining the neuron vs glial cell composition of cultures

The ratio of neuronal cells to glia was determined by counting the number of pan-neuronal and GFAP -positive cells, respectively, under a confocal fluorescent microscope. As described previously, when cells were initially added to cover slips following their dispersal, a drop was placed in the centre of each coverslip. As a

result, there is a non-uniform distribution and density of cells, with the highest density of cells appearing in the centre of the coverslip and receding outwards towards the perimeter of the initial cell droplet (see Suppl. Fig. 1.). As such, in order to obtain an accurate representation of overall cell numbers, a central point was chosen and three randomised points radiating from the middle to the edge of the immunostained region of each coverslip were selected for counting (such regions contained an average of 16 cells in an area of approximately 200 μm^2). Compositional analysis was conducted upon coverslips fixed at 2, 5, 8, 11, 14, 17 and 20 days *in vitro* and from three separate culture preparations (from three separate mouse litters), harvested from 5 day old mice.

2.5.7 Calcium Imaging

Intracellular calcium measurements from the soma of neurons were carried out as previously described (Rae et al., 2000). Briefly, using a conventional fluorescence imaging system (Cairn Life technologies), neurons that had been preloaded for one hour with the single wavelength, intensity modulating calcium indicator, fluo-2 AM (excitation at 488 nm, emission at 550 nm) (4 µM), were imaged. Images were acquired at one second intervals using the open source imaging software, Winfluor (John Dempster, University of Strathclyde, Scotland) via an Olympus BX50 WI microscope (20× objective). Winfluor calculated fluorescence values for each pixel in the frame, after subtraction of background fluorescence intensities. Representative traces were created off line using Graphpad Prism.

3. Results

3.1 Production and characterisation of postnatal primary mouse hippocampal cultures

We have consistently produced postnatal hippocampal mouse cultures from animals between 3 – 5 days old, which are routinely used for calcium imaging experiments. Such cultures contain healthy, functional cells which respond appropriately to physiological stimuli (see 3.2 Calcium imaging experiments). Cultures display normal neuronal morphology, arborisation, synaptic connections and inter-cellular associations from 2 days *in vitro* (DIV) onwards, evident from both bright field images (Fig. 1 & 2) and from immunocytochemical images (Fig. 3 & 4 & Suppl. Fig. 2 for corresponding phase contrast images) which were stained with a pan-neuronal marker and with glial fibrillary acidic protein (GFAP) and synapsin I antibodies. In order to characterise the development and composition of such cultures *in vitro* we examined the neuron to glial cell ratio after fixing cultures at 2, 5, 8, 11, 14, 17 and 20 DIV (Fig. 5). Although there is a steady decline in the numbers of both glia and neurons with increasing DIV, the neurons which do remain respond to standard physiological stimuli for up to one month after initial culturing (*e.g.* Suppl. Fig. 3).

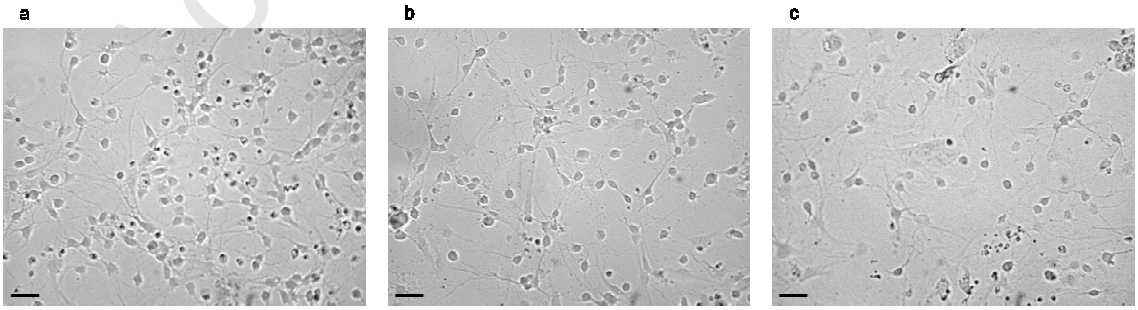

Figure 1. Bright field (DIC) images showing cultured postnatal mouse hippocampal neurons generated from 3 (a), 4 (b) and 5 (c) day old mice. All images were captured at 2 days *in vitro*. Scale bar 30 µm.

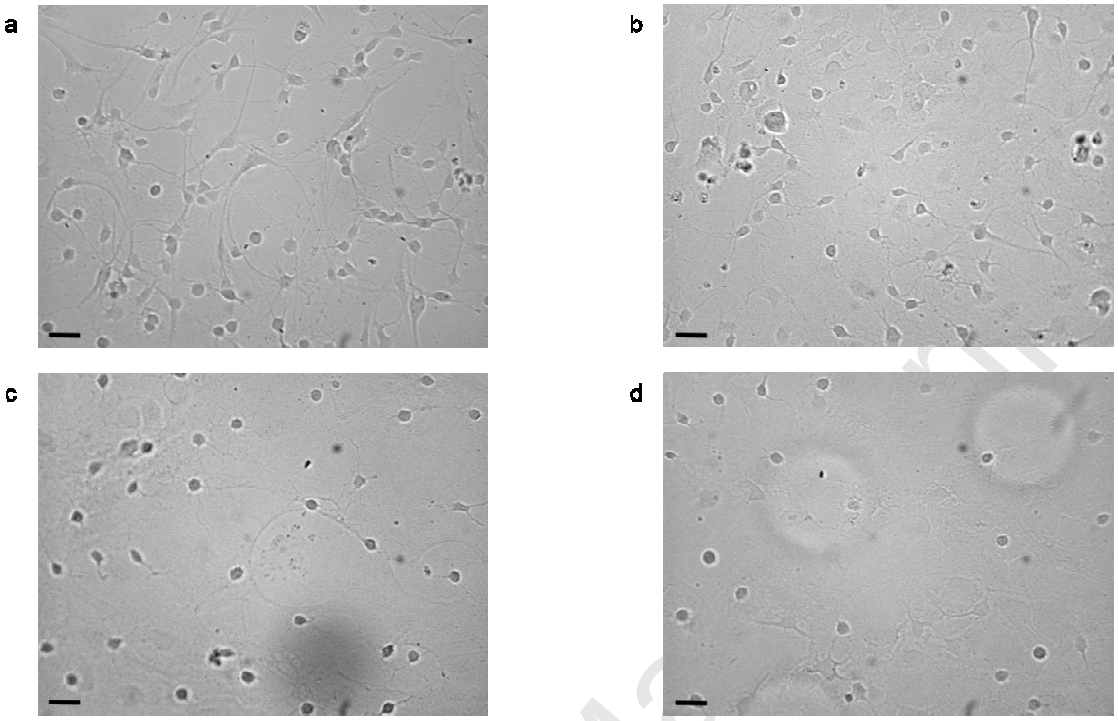

Figure 2. Bright field (DIC) images of cultured mouse hippocampal neurons taken at progressively older days *in vitro,* 2 (a), 4 (b), 10 (c), 13 (d) All cultures were generated from mice aged between 3 - 5 days of age. Scale bar 30 µm.

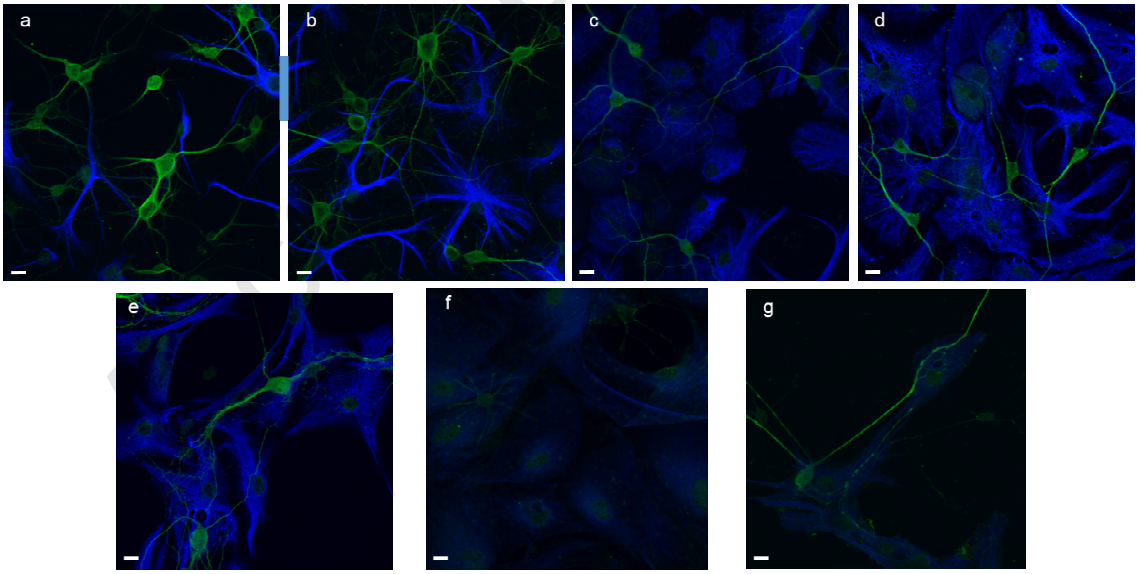

Figure 3. Immunostaining of hippocampal cultures for neuronal and glial markers. Images were captured from cultures fixed at progressively older days *in vitro* 2 (a), 5 (b), 8 (c), 11 (d), 14 (e), 17 (f) and 20 (g). Neuronal marker, pan-neuronal stain containing NeuN, βii-tubulin, NF-H and MAP-2 (green). Glial marker, GFAP (blue). All cultures were generated from 5 day old mice. Scale bars, 10 µM.

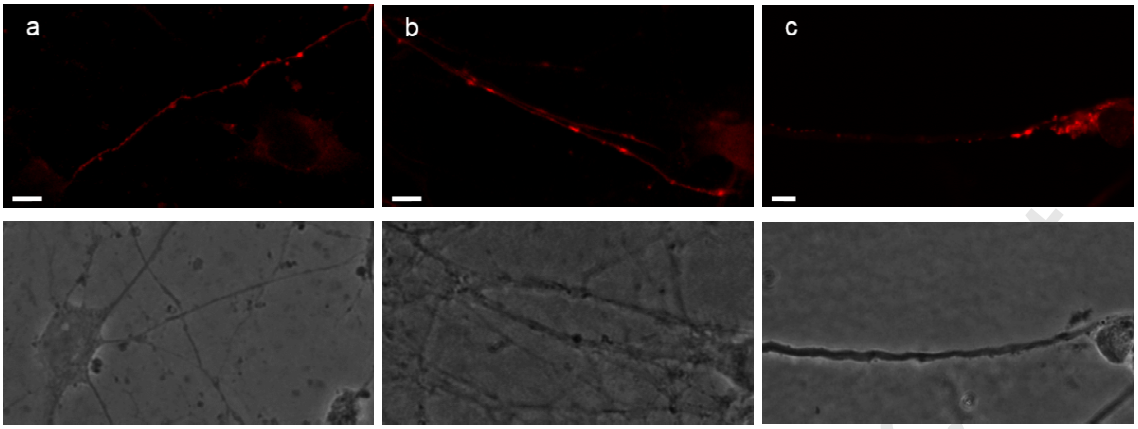

Figure 4. Immunostaining of hippocampal cultures for synaptic marker (synapsin I, red) with corresponding phase contrast images. Images were captured from cultures fixed at progressively older days *in vitro*, 2 (a), 10 (b) and 20 (c). All cultures were generated from 5 day old mice. Scale bars, 5 µM.

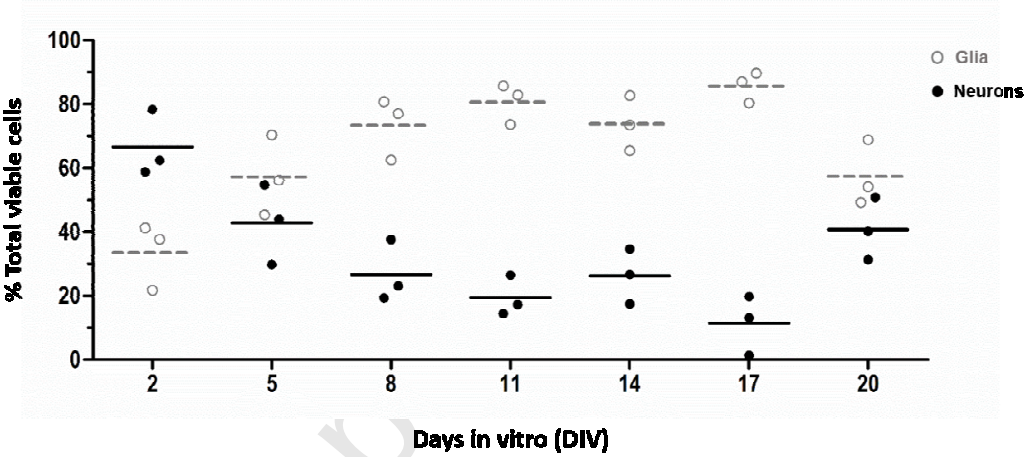

Figure 5. Graphical representation of the neuron *versus* glial cell composition of postnatal mouse hippocampal cultures at progressively older days *in vitro* (DIV). The percentage of viable cells which are glia (grey, open circles) or neurons (black, closed circles) are presented at 2 – 20 DIV. All cultures were generated from 5 day old mice.

3.2 Calcium imaging experiments

Cultured neurons harvested from 3 - 5 day old mice are usually utilised up to fourteen days *in vitro* for calcium imaging experiments (Kaar and Rae, 2016, 2014). However, some experiments have also been carried out using neurons which had been cultured for approximately one month in order to test long-term cell function

(Suppl. Fig. 3). In the course of our calcium imaging investigations we have utilised various compounds which healthy and functional cultured hippocampal neurons would be expected to respond to. For example, we have conducted experiments in which these neurons have responded to physiologically relevant stimuli such as 15 mM and 50 mM K^+-containing HBSS (Fig. 6 a & b) (which depolarise neurons and thereby activate voltage-gated calcium channels (VGCCs) on the neuronal plasma membrane); the specific group I metabotropic glutamate receptor (mGluR) agonist, (S)-DHPG (50 µM), the muscarinic acetylcholine receptor agonist, carbachol (10 µM) and caffeine (20 mM), which all evoke calcium release from the endoplasmic reticulum (ER) (Fig. 6 c, d and f, respectively);, as well as the ionotropic glutamate receptor agonist, NMDA (1 µM; Fig. 6 d). All compounds were added to the superfusate.

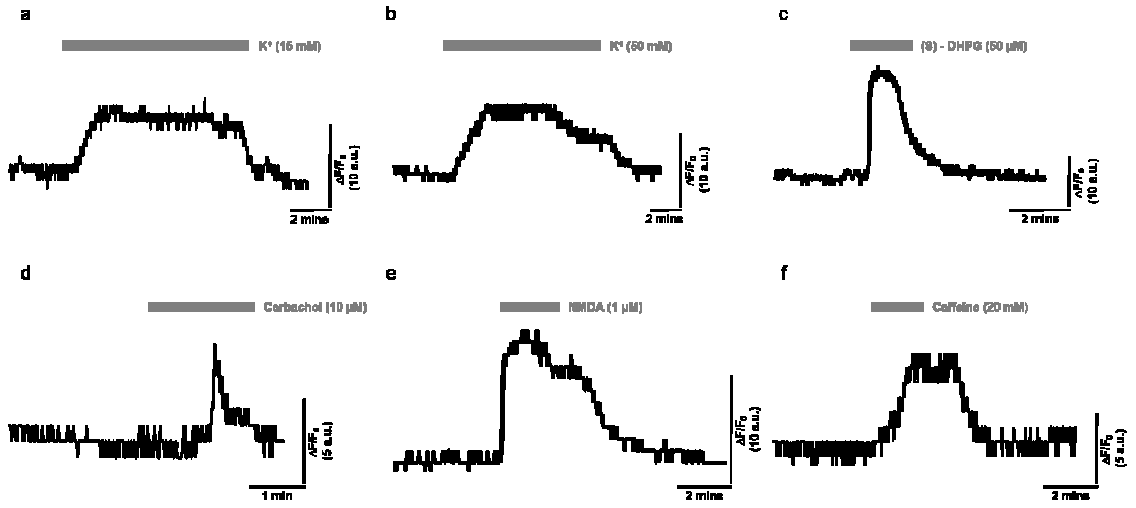

Figure 6. Representative traces showing changes in somatic [Ca^{2+}] levels within a cultured mouse hippocampal neuron in response to selected physiological stimuli. Representative traces display responses to the application of: K^+ (15 mM) (a), K^+ (50 mM) (b), (S)-DHPG (50 µM) (c), Carbachol (10 µM) (d), NMDA (1 µM) (e) and Caffeine (20 mM) (f). All compounds were added to the superfusate. All neurons were generated from 3 – 5 day old mice and imaged after 2 – 14 days *in vitro*.

4. Discussion

The study of postnatal neurons, and therefore the use of postnatal cultures, is important for the investigation of chronic and/or age-dependent conditions such as neurodegeneration. Furthermore, as mentioned earlier, the importance and widespread use of transgenic *mouse* models, but the inherent difficulty in culturing postnatal mouse neurons relative to embryonic tissues (Brewer and Torricelli, 2007) or those of the rat (Beaudoin et al., 2012), underpins the need for an easily applicable and effective postnatal mouse culture protocol.

Herein we have described a novel, simplified protocol for the cost-effective production of primary cultured neurones from postnatal mouse hippocampal tissue, which remain viable for a minimum of one month post-culture. Hippocampal neurons within such cultures display normal development and arborisation, observable cellular inter-connection (including synapses) and association with glial cells. The utilisation of either “pure” neuronal cultures, mixed cultures with or without glial feeder layers is dependent upon the particular experiments one wishes to conduct. However, given the role that glia play in supporting neuronal function, signalling, synaptic plasticity, *etc.* (Araque and Navarrete, 2010; Auld and Robitaille, 2003; Shaham, 2005) the presence, or otherwise, of glial cells in neuronal cultures will almost certainly impact upon both culture development and phenotype. We have opted not to use glial inhibitors, partly because they negatively impact upon culture viability and synaptic development (Beaudoin et al., 2012; Pfrieger and Barres, 1997; Ullian et al., 2001) but also because we feel the presence of such cells is more reflective of the physiological environment which we are trying to model with these cultures. However, depending upon the specific research question one wishes to

545 investigate, the medium components suggested herein could be fine-tuned to
546 generate the particular final cellular composition one desires.

547 Importantly, the preparation and long-term survival of our neuronal cultures was
548 independent of any requirement for either proprietary media and/or supplements
549 (Ahlemeyer and Baumgart-Vogt, 2005; Beaudoin et al., 2012; Brewer and Torricelli,
550 2007; Kivell et al., 2000; Nunez, 2008; Zhang et al., 2006) or complex and/or lengthy
551 procedures such as glial feeder layers (Kaech and Banker, 2006) and gradient
552 isolation techniques (Brewer and Torricelli, 2007; Lee et al., 2009). Significantly,
553 even without using these 'essential' steps in the preparation of hippocampal cultures,
554 we have routinely utilised our neurons (between two and fourteen days *in vitro*) for
555 both calcium imaging, and whole-cell patch-clamp recording with simultaneous
556 calcium imaging experiments, in which normal physiological responses have been
557 recorded, indicating that the neurons remained viable and responsive even for this
558 extended time in culture.

559 Contrary to previous studies such as that of Eide and McMurray (2005), we have
560 found that the manner in which the tissue is treated following incubation with tissue
561 proteases, specifically the steps of trituration and subsequent re-suspension after
562 supernatant centrifugation, is absolutely critical to ensuring the production of
563 consistent and successful cultures. In this respect, mouse neurons seem to be much
564 more vulnerable and susceptible to mechanical stress than postnatal rat and
565 embryonic neurons at this point in the protocol. Thus, by using the novel trituration
566 technique described here, whereby we have reduced to an absolute minimum the
567 total number of triturations (<4) of the hippocampal tissue, and re-suspended the cell
568 pellet only using with finger vortexing, we believe that we have minimised the
569 mechanical stress experienced by the neurons. In turn, this results in the production

of a cell suspension containing a very high percentage of viable cells (85 - 90% viability as determined by trypan blue protocol).

To the best of our knowledge only three other papers have demonstrated effective postnatal hippocampal culture from mouse nervous tissue, but all utilised the aforementioned proprietary media and supplements. The method described by Eide and McMurray (2005) using striatal and cortical mouse tissue is particularly impressive given the age of the animals involved, 1–1.5 years old. Unfortunately, it is unclear how these achieved this successful outcome as the paper lacks methodological detail about the protocol that was employed to prepare the neurons. Although the methods employed by both Beaudoin *et al.* (2012) and Brewer *et al.* (2007), using early postnatal (P0–P1) and adult mouse hippocampal tissue respectively, are well described and imply that good quality cultures can be produced, they are much more laborious and expensive than the protocol described here. Therefore, our technique would appear to offer several advantages over these aforementioned studies, in that the methodology is simple and efficient, cultures can be produced from older animals (routinely 3 - 6 day old mice, but numerous cultures have been produced up to day 14 (unpublished data) and the protocol has relatively lower costs of production (given the use of non-proprietary supplements).

5. Conclusions

In summary, we present here a simplified, economical and reliable method for consistent production of primary hippocampal cultures from postnatal mouse hippocampal tissue. Using this technique, we are able to reliably and reproducibly produce hippocampal neuronal cultures of a consistently high standard. Furthermore, the neurons within these cultures, in addition to displaying normal characteristics of healthy neurons, also exhibit consistent and reproducible responses to physiological stimuli. We propose that this method could also be utilised to produce cultures of other postnatal mouse neuronal tissues.

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Highlights

1. Efficient and cost-effective method for postnatal mouse hippocampal culture
2. Novel trituration technique, simplified methodology and non-proprietary reagents
3. Normal neuronal morphology and appropriate responses to physiological stimuli
4. Neurons remain functional in an incubator for over one month

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